The hepatitis B virus-X protein (HBx), known as a multifunctional protein, is involved in the activation of a wide variety of different enhancer/promoter functions by direct or indirect interactions with transcription factors (1), activation of signal transduction pathways (2), sensitization of cells to apoptosis (3), loss of cell cycle checkpoints (4), induction cell growth arrest (5), and modulation of proteolytic degradation pathways (6). HBx does not activate apoptotic signaling, although, is likely to sensitize hepatoma cells to apoptotic signaling, which is dependent on reactive oxygen species. Increased intracellular lipid peroxidation in HBx transgenic mice demonstrated that oxidative injury occurs as a direct result of HBx expression. Therefore, we conclude that mitochondrial dysfunction is a crucial pathophysiological factor in HBx-expressing hepatoma cells and provides an experimental rationale in the investigation of mitochondrial dysfunction in rapidly renewed tissues, as in hepatocellular carcinomas.

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after HBV infection. In this report, we show that HBx alters mitochondrial functions; we also demonstrate down-regulation of enzymes in electron transport in oxidative phosphorylation, sensitization of the ΔΨm to inhibitors of electron transport, and sensitization of hepatoma cells to apoptotic stimuli. These results together with the observed increase in the cellular abundance of ROS with a consequential increase in cellular lipid peroxidation shed new light on the physiological significance of the HBx effect on mitochondria which can contribute to liver disease associated with HBV infection.
HBx Effect on Liver Mitochondrial Function and Physiology

### EXPERIMENTAL PROCEDURES

**Cell Lines and Materials**—The open reading frame of HBx of pHBV-D plasmid (19) was cloned into the pMAMneo-X expression vector (Clontech) and named pMAMneo-X. Hepatoma cells and HBx-transfected hepatoma cells (20) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (JBI, Daegu, Korea), 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. The in situ cell death detection kit for the TUNEL assay was from Roche Applied Science. The anti-cytochrome c monoclonal antibody was from Pharmingen (San Diego). The monoclonal antibody against the DNA repair enzyme, poly(ADP-ribose) polymerase (PARP), was from Enzyme System Products (Dublin, CA). Tetramethylrhodamine methyl ester (TMRM), 2’,7’-dichlorofluorescein diacetate (DCFH-DA), and hydroethidine (HE) were from Molecular Probes, Inc. (Eugene, OR). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG was from Jackson ImmunoResearch Laboratory (West Grove, PA). Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), glutathione, Hoechst 33258, propidium iodide, staurosporine, and vitamin K3 (VK3) were from Sigma.

**Differential Display-PCR Analysis**—Differential display-PCR analysis for the detection of differentially expressed genes in HepG2 and HBx-transfected HepG2-3X cells was performed essentially by the method of Liang and Pardee (21) using the RNAmap kit according to the manufacturer’s instructions as described previously (22).

**Submitochondrial Particle Preparation and Immunoblotting**—HepG2 and HepG2-1X, 3X, and 4X cell mitochondria were prepared according to Bhat et al. (22) and stored in 250 mM sucrose suspension at −20 °C. Submitochondrial particles (SMP) were prepared essentially as described by Pedersen et al. (24), except that EDTA was omitted from the sonication medium. Briefly, frozen mitochondrial suspension was thawed and diluted with 250 mM sucrose to a concentration of about 20–30 mg/ml. The mitochondria were then subjected to sonication for 2 min at the maximal output with a Branson sonifier in an ice bath under a nitrogen stream. The suspension was decanted and centrifuged at 105,000 g for 50 min. The resulting pellet, consisting of SMP, was washed and suspended in 250 mM sucrose. Proteins were determined by the usual biuret method using bovine serum albumin as standard. For immunoblot analysis, proteins were run on 12% SDS-urea gel and transferred to nitrocellulose membrane. Transferred proteins were probed with cytochrome oxidase I, II, III, IV, and VI-b mouse monoclonal antibodies (Molecular Probes). Reactive bands were detected using the enhanced chemiluminescence (ECL) Plus™ detection reagent (Amersham Biosciences).

**Measurement of Enzyme Activities**—NADH-CoQ oxidoreductase (complex I) activity in SMP from HepG2 and HBx-expressing HepG2 cell lines was assayed by measuring the NADH disappearance at 450 nm as described previously (25). Briefly, 10–15 μg of SMP was added to 300 μl of the assay medium (50 mM KCl, 10 mM Tris-HCl, 7.4, 1 mM EDTA, 2 mM KCN, pH 7.4), and 75 μM NADH and electron acceptor 40 μM decylubiquinone were added for enzyme activity assay. Oxidation of NADH (NADH disappearance) was followed after A450 nm. After adding 40 μM decylubiquinone, and the values were expressed as nmol/min/mg of SMP. Succinate:ubiquinone oxidoreductase (complex II) activity was measured by following the reduction of ubiquinone observed at 278 nm using an extinction coefficient of 14.7 cm−1 mM as described (26). 30 μM decylubiquinone was used as the electron acceptor. Ubiquinone-cytochrome c oxidoreductase (complex III) activity was measured using diode array spectrophotometer (Agilent 8453, Palo Alto, CA) by following the increase in reduced cytochrome c absorbance at 540 nm. The SMP sample (10 μg) was added to 3 ml of the assay mixture (50 μM ferricytochrome c in 50 mM phosphate buffer, pH 7.2), and the reaction was started by the addition of 30 μM decylubiquinone. The activity was calculated using an extinction coefficient of 19.1 cm−1 mM for reduced cytochrome c. The specific activity of the enzyme is expressed as nmol of cytochrome c reduced/min/mg of SMP. Cytochrome c oxidoreductase (complex IV) activity in SMP was assayed by measuring the rate of oxidation of ferricytochrome c at 550 nm. The reaction medium consisted of 0.1% Triton X-100, 1 mM sodium phosphate, pH 7.0, 1% sodium cholate, 80 μM ferricytochrome c, 1 mM EDTA, and 1–2 μg of SMP protein in a total volume of 1 ml. Ferricytochrome c concentrations were determined using an extinction coefficient of 21.1 cm−1 mM at 550 nm and the values expressed as nmol/min/mg of SMP. ATP synthase (complex V) activity in SMP was assayed by measuring ATP formation as described previously (27).

**Flow Cytometric Analysis of the Mitochondrial Membrane Potential**—HepG2 and HBx-transfected HepG2 cells were plated on 100-mm Petri dishes. After a 48-h incubation with complete medium, the cells were treated with FACSscan flow cytometer according to the instructions of the manufacturer. Then, cells were trypanized, transferred to 15-ml Falcon tubes, and centrifuged at 1,200 rpm for 3 min. The supernatant was removed, and the pellet was washed with 5 ml of PBS. The cells were resuspended in complete medium containing the fluorochrome TMRM at a final concentration of 150 nM. After incubation for 30 min at 37 °C, the emission fluorescence was measured using the FL2 channel (57,526 nm) of a FACSscan flow cytometer at 549 nm (excitation) and 573 nm (emission). Values for mitochondria with depleted ΔΨm were determined by simultaneous treatment of cells with the protonophore CCCP (final concentration, 100 nM) and TMRM.

**Measurement of Lipid Peroxidation Products**—Appropriate cell culture (5 × 10⁴ cells) lysates were prepared by sonication and stored at −70 °C after the addition of 5 mM/liter butylated hydroxytoluene (Sigma). 4-Hydroxynonenals and malondialdehyde were measured in these homogenates using a commercial assay kit (LPO-586; OXIS International, Inc., Portland, OR). The protein concentration was determined by a Coomasie assay (Pierce).

**Detection of DNA Fragmentation**—After 48-h incubation, HepG2 and HBx-transfected HepG2 cells were subjected to sodium and stored at −20 °C. Cells were washed with PBS and 100 μl paraformaldehyde (4% in PBS, pH 7.4) for 30 min at room temperature. After washing once with PBS, cells were permeabilized with 100 μl of permeabilization buffer (0.1% Triton X-100, 0.1% sodium citrate) for 2 min at 4 °C. The TUNEL reaction was carried out by incubating cells in a moisture chamber for 1 hr at 37 °C with TUNEL reaction mixture (Roche Applied Science) in a total reaction volume of 50 μl. After washing three times, samples were analyzed under a fluorescence microscope. Hoechst staining was done according to the manufacturer’s instructions as described previously (28).

**Detection of Bax Content**—For the detection of Bax, mitochondrial

### TABLE I

<table>
<thead>
<tr>
<th>Complex</th>
<th>HepG2</th>
<th>HepG2</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (NADH-CoQ oxidoreductase)</td>
<td>827</td>
<td>292</td>
</tr>
<tr>
<td>II (succinate:ubiquinone oxidoreductase)</td>
<td>1,862</td>
<td>1,736</td>
</tr>
<tr>
<td>III (ubiquinone-cytochrome c oxidoreductase)</td>
<td>1,726</td>
<td>632</td>
</tr>
<tr>
<td>IV (cytochrome c oxidoreductase)</td>
<td>2,376</td>
<td>897</td>
</tr>
<tr>
<td>V (ATP synthase)</td>
<td>3,824</td>
<td>1,523</td>
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</table>

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>HepG2 IX</th>
<th>HepG2 3X</th>
<th>HepG2 4X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I</td>
<td>313</td>
<td>275</td>
<td>47</td>
</tr>
<tr>
<td>Complex II</td>
<td>1,829</td>
<td>1,832</td>
<td>262</td>
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<tr>
<td>Complex III</td>
<td>718</td>
<td>536</td>
<td>136</td>
</tr>
<tr>
<td>Complex IV</td>
<td>976</td>
<td>732</td>
<td>128</td>
</tr>
<tr>
<td>Complex V</td>
<td>1,231</td>
<td>1,421</td>
<td>432</td>
</tr>
</tbody>
</table>

**Rotenone** (1.25 μM) | **TTPA** (12.5 μM) | **Antimycin A** (12.5 μM) | **Azide** (500 μM) | **Oligomycin B** (6.25 μM) |

| nmol · min⁻¹ · mg⁻¹ SMP | nmol · min⁻¹ · mg⁻¹ SMP | nmol · min⁻¹ · mg⁻¹ SMP | nmol · min⁻¹ · mg⁻¹ SMP | nmol · min⁻¹ · mg⁻¹ SMP | nmol · min⁻¹ · mg⁻¹ SMP | nmol · min⁻¹ · mg⁻¹ SMP | nmol · min⁻¹ · mg⁻¹ SMP |

| Complex I | 827 | 292 | 313 | 275 | 47 |
| Complex II | 1,862 | 1,736 | 1,829 | 1,832 | 262 |
| Complex III | 1,726 | 632 | 718 | 536 | 136 |
| Complex IV | 2,376 | 897 | 976 | 732 | 128 |
| Complex V | 3,824 | 1,523 | 1,231 | 1,421 | 432 |

**Activities**
and cytosolic fractions (15 μg of protein) were analyzed by SDS-PAGE in a 15% gel with equal amounts of protein loaded into each well as determined by the Bradford assay (Bio-Rad). Kaleidoscope prestained standards (Bio-Rad) were used to determine the molecular mass. The gels were then electroblotted onto polyvinylidene difluoride transfer membranes (Amersham Biosciences). For the detection of Bax, anti-mouse IgG against Bax (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the primary antibody and horseradish peroxidase-labeled goat anti-mouse IgG as the secondary antibody. ECL was used for detection of Bax expression.

Analysis of PARP Cleavage and Immunoblotting—For Western blot analysis of PARP cleavage, both floating and attached cells were rinsed in cold PBS, pH 7.4, and then collected with 2 ml of lysis buffer (62.5 mM Tris, pH 6.8, 8 M deionized urea, 10% glycerol, 2% SDS, and protease inhibitors). The cells were then sonicated on ice for 20 s. After the addition of loading buffer, the samples were incubated at 65 °C for 15 min, and equal amounts of protein were resolved on a 7.5% SDS-polyacrylamide gel. Immunoblotting for PARP cleavage was performed using a monoclonal antibody that specifically detects human PARP at a 1:2,000 dilution. Visualization of the signal was by ECL.

Production of HBx Transgenic Mice—The HBx transgenic mice generated using a pHEX-1 expression vector were described previously (29).

Immunohistochemical Staining Analysis—The immunohistochemical staining for lipid peroxidation products and HBx protein was basically followed as described previously (29). For the primary antibodies, a HBx antibody (30) and a malonyl dialdehyde-specific antibody (MDA-Ab) (31) for lipid peroxidation products were used. For the secondary antibody, a universal secondary antibody (DAKO, Foster City, CA) was used. Sections were labeled with peroxide-conjugated streptavidin (DAKO) for 10 min, incubated in diaminobenzidine, and washed in Immuno/DNA buffer solution (Research Genetics, Huntsville, AL). Finally, the slides were counterstained with Mayers hematoxylin, washed in distilled water, and mounted with universal mount (Research Genetics). The specificity of immunohistochemical staining was verified using PBS in place of primary antibodies. Negative controls always gave negative staining of the tissues.

RESULTS

The HBx Protein Down-regulates Mitochondrial Enzymes Involved in Oxidative Phosphorylation—Human hepatoma cell lines expressing the HBx protein in HepG2 (1X, 3X, and 4X) and HepG3B (6X and 8X) cells were established and characterized (20). By using these cell lines we screened the genes that are expressed differentially by HBx. We employed the differential display-PCR technique, using the RNAmap kit (GenHunter Corp, Brookline, MA) according to the manufacturer’s instructions. Total RNA from HepG2 and HepG2-3X cell lines was reverse transcribed into cDNA with three anchored oligo(dT) primers. Selected portions were amplified by PCR with the first strand cDNA primers and with eight different kinds of 5'-arbitrary primers as described in the RNAmap kit. The PCR products were separated on a 1.2% agarose gel. Visualization of the signal was by ECL.

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chrome c oxidase, ATP synthase subunit 6, and NADH-CoQ oxidoreductase. Three different cDNA fragments (NADH-CoQ oxidoreductase, cytochrome c oxidase I, and ATP synthase subunit 6) as mentioned above were used as probes for Northern blot analysis containing total RNA of the HepG2, Hep3B, and HBx-transfected HepG2, Hep3B cells (HepG2-1X, 3X, and 4X; Hep3B-6X and 8X). As shown in Fig. 1A-I, mRNAs of the expected size of each product were detected. The intensity of the signals of each mRNA was 50–80% lower in HBx-transfected cells than in the control (Fig. 1A-I). Among the five enzyme complexes involved in steps of oxidative phosphorylation in mitochondria, we isolated three enzymes involved in electron transport. Because there are two more complexes involved in oxidative phosphorylation (complex III, ubiquinone-cytochrome c oxidoreductase; complex II, succinate:ubiquinone oxidoreductase), we did Northern blot analysis using respective gene probes as shown in Fig. 1A-II. The intensity of expression of ubiquinone-cytochrome c oxidoreductase (complex III) in each HBx-transformed cell line was lower, whereas the succinate:ubiquinone oxidoreductase (complex II) signal was unchanged compared with control HepG2 cells (Fig. 1A-II). The pYKM ND-5 vector, which expresses the 70-amino acid N-terminal deleted mutant form of HBx and showed defects in transactivating various promoters (32), was used for transformation of the HepG2 cell line. The resulting cell line HepG2-ND5X was used as a control. The enzyme activities of complexes I–V were measured on SMP from HepG2, HepG2-1X, 3X, and 4X cell lines (Table I). Enzyme activity analysis also showed that HBx down-regulates the enzyme activities involved in oxidative phosphorylations (complexes I, III, IV, and V) (Table I). These results further confirmed that HBx down-regulates the enzymes that are specifically involved in electron transport and proton translocations (complexes I, III, IV, and V), whereas it does not down-regulate complex II, succinate: ubiquinone oxidoreductase, which is involved in electron transport but not in proton translocations in mitochondria. The
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research on the molecular mechanism of HBx down-regulation of complexes I, III, IV, and V but not complex II has progressed in this laboratory. To verify the specificities of the enzyme activities we treated inhibitors of electron transport or uncoupling of oxidative phosphorylations in each complexes and found that inhibitors specifically abolish the enzyme activities as shown in Table I. Next we questioned whether the same reduction in expression was observed in cytochrome c oxidase subunits other than subunit I, so the expression of four subunits (subunits II, III, IV, and VI-b) in HepG2, Hep3B, and HBx-transfected cells was analyzed. Similar to Fig. 1A, mitochondrial encoded cyclooxygenase (COX) subunits (COX I, II, and III) showed consistent underexpression in HBx-transfected cells (Fig. 1B-I), whereas the levels of nuclear encoded subunits IV and IV-b, among 10 nuclear encoded subunits, did not show differences in the levels of the control (HepG2 and Hep3B) (Fig. 1B-II). These results also revealed another interesting result showing that only the mitochondrially encoded subunit genes are down-regulated by HBx, whereas nuclear encoded subunit genes are not affected by HBx. We did the Western blotting analysis using COX I, II, III, IV, and VI-b antibodies to confirm the Northern results as shown in Fig. 1C. The Western analysis results also showed that mitochondrially encoded subunit genes are down-regulated by HBx, whereas nuclear encoded subunit genes are not affected by it. Immunohistochemical analysis of COX I (encoded by mitochondrial DNA) and COX IV (encoded by nuclear DNA) showed that both COX I and COX IV were expressed in HepG2 cells, whereas COX I expression was decreased dramatically in HepG2-3X; this further confirmed that HBx down-regulates mitochondrial encoded COX subunits at the protein level (Fig. 1C, I and II).

HBx Expression Increases Cellular ROS Productions in Hepatoma Cells—Because we demonstrated that HBx down-regulates genes involved in oxidative phosphorylation at mitochondria, a major ROS generator, we questioned whether HBx induces ROS production in HBx-transfected cells. We detected intracellular ROS production by staining with the hydrogen peroxide-sensitive fluorescent dye DCFH-DA, and the H$_2$O$_2$ levels were measured using a FACScan flow cytometer with excitation and emission settings of 488 and 530 nm, respectively, and with a laser scanning confocal microscopy (Fig. 2, A and B). As shown in Fig. 2, A and B, the DCF fluorescence displayed an increase in HBx-transfected HepG2 cells, whereas GSH or pyruvate treatment showed a decline in the fluorescence to the basal level (Fig. 2B). We next quantified the total cellular lipid peroxidation products in extracts from these cells (Fig. 2C). This information provides a measure of the consequences of oxidative stress because peroxidation of lipids is a result of elevated cellular ROS. The HBx expression resulted in a significant increase in the abundance of lipid peroxide products in both HepG2-3X and Hep3B-6X cells (Fig. 2C). This result confirms that HBx protein expression induces oxidative injury in these cells. The $\Delta\Psi$m in HepG2 and HBx-transfected HepG2 cells was measured using the fluorochrome TMRM. For the positive control, 50 $\mu$M CCCP was added. Cells were analyzed with a FACScan flow cytometer. As shown in Fig. 2D, there was no difference in the $\Delta\Psi$m between HepG2 and HBx-expressing HepG2-3X cell lines. The other HBx-expressing cell lines from HepG2 and Hep3B cells also showed no difference in the $\Delta\Psi$m (data not shown). These results confirm that HBx protein expression by itself did not induce change in $\Delta\Psi$m, although it induces ROS production, resulting in oxidative injury in these cells.

Mitochondria Are the Source of HBx-induced ROS—There are many reports that ROS production is increased when the electron flow in respiratory chain is impaired, especially complex I in oxidative phosphorylations (33, 34). It has been shown that complex I is a major ROS-generating site where inhibition to a small degree by complex I inhibitor, rotenone, is sufficient in ROS production, whereas complexes II, III, and IV could be inhibited by about 70% without an increase in ROS production (35). A recent report also showed that increased reduction of ubiquinone and partially reduced ubisemiquinone at complex I appear to react directly with O$_2$ and to be a major source of oxygen free radicals (36). The mitochondrial component of ROS production can be inhibited quantitatively by diphenyldionium (DPI), an irreversible inhibitor of flavoenzymes which blocks electron transport through mitochondrial complex I and thus prevents mitochondrial ROS production. Fig. 3A-II shows that 100 $\mu$M DPI treatment entirely inhibited the increase in the cellular ROS level by HBx in HBx-transfected HepG2-3X cells, confirming that mitochondria are the source of HBx-induced ROS. Under these conditions there was no effect of DPI on cell death as assessed by trypan blue staining (data not shown). DPI-treated HepG2 cells were used as a control (Fig. 3A-I). Other mitochondrial respiratory chain inhibitors, rotenone and antimycin A, were known to be a cause of ROS production in control cells (36) and thus were not used for determining the source of HBx-induced ROS. We also showed that 12.5 $\mu$M TTFA, a mitochondrial electron transport chain complex II inhibitor, did not show any inhibition of the produc-

Fig. 4. Hepatic lipid peroxidation in transgenic mice expressing HBx proteins. Immunohistochemical staining for lipid peroxidation products (MDA adducts, see “Experimental Procedures”) in HBx transgenic mice (C and D) and control mice (A and B) is shown. Positive reactions in immunohistochemical staining for lipid peroxide are found in the non-tumor region (C) and tumor region (D) of HBx transgenic mouse liver. Immunohistochemical staining for lipid peroxidation is negative in the liver of HBx transgenic mice without MDA-Ab treatment (A) and in the liver of nontransgenic mice with MDA-Ab treatment (B). HBx proteins are expressed in the liver of HBx transgenic mice that did not have a hepatic tumor (E) and in the tumor region (right) and non-tumor region (left) of the HBx transgenic mice (F). The magnification of each photograph is indicated. Samples were counterstained with hematoxylin in A–E and were not counterstained in F.
tion of ROS in HepG2-3X cells (Fig. 3A-III). The results of Fig. 3A were quantified and are given in Fig. 3B as the relative ROS production in each case. Because these results suggest the possibility of a direct effect of HBx on mitochondrial function, the spatial relationship between HBx and mitochondria was determined by fluorescence microscopy. Fig. 2C shows the relationship between indirect immunofluorescence for HBx protein (green) and mitochondria (stained with Mito Tracker Red). DAPI was used for nuclei (blue) staining. There were numerous areas of overlap between mitochondria and the HBx protein, particularly in the perinuclear region, which appears as yellow in HepG2-3X cells. HepG2 cells that do not show any HBx staining, therefore, do not show any merge on mitochondria, and HBx protein was used as a control. These results together with those of Fig. 3, A and B, confirm that mitochondria are the source of HBx-induced ROS production.

**HBx-induced Lipid Peroxidation in HBx Transgenic Mice**—As a secondary indication of mitochondrial dysfunction induced by HBx, we analyzed the lipid peroxidation products (MDA adducts) from hepatocytes of HBx transgenic mice (Fig. 4). The negative controls with no MDA-Ab treatment of HBx transgenic mice (A) or MDA-Ab treated HBx nontransgenic mice (B) all showed negative immunohistochemical staining. Immunohistochemical stains for lipid peroxidation revealed positive reactions on the liver of non-tumor region (C) in 19 among 21 HBx transgenic mice. Hepatic tumors were found in the 7 mice among 21 HBx transgenic mice. All of tumor regions (D) in 7 tumors bearing HBx transgenic mice were positive reactions in immunohistochemical staining for lipid peroxidation. These results suggest that HBx, although it does not form tumors, induces liver cell injury by inducing ROS and eventually leads to the formation of lipid peroxide products in liver cells. To verify the HBx expression in HBx transgenic mice, immunohistochemical staining for HBx protein was used in this experiment. The HBx-positive hepatocytes are found in the liver of 6-month-old male HBx transgenic mice that did not have hepatic tumors (E). The HBx-positive cells were also found in the tumor region and non-tumor region of the 11-month-old male HBx transgenic mice (F).

**HBx Sensitizes the Cells to Inhibitors of the Electron Transport Chain in Oxidative Phosphorylation**—Our results showed that HBx expression did not change the $\Delta \Psi m$, although it caused dramatic changes in the expression and function of genes involved in mitochondrial respiration and ROS productions. Because electron transport is essential for production and maintenance of the $\Delta \Psi m$, we next investigated the effect of blocking electron transport on the $\Delta \Psi m$ in HBx-transfected cells. HepG2 and HBx-transfected HepG2 (HepG2-3X) cells were thus treated with 1.25 $\mu$M rotenone, the inhibitor of mitochondrial enzyme complex I; the initial step in electron transport, 12.5 $\mu$M TTFA, the inhibitor of complex II; 12.5 $\mu$M antimycin A, the inhibitor of complex III; 500 $\mu$M azide, the inhibitor of complex IV; and 6.25 $\mu$M oligomycin, the inhibitor of complex V. For the positive control, valinomycin, the K$^+$ ionophore that uncouples oxidative phosphorylation, resulting in collapse of the $\Delta \Psi m$, was used. Both cell lines were treated with the six different inhibitors and stained with TMRM by flow cytometry. The concentration of each inhibitor was chosen as suggested (37). As illustrated by the representative cytofluorometric analysis shown in Fig. 5, the $\Delta \Psi m$ of HBx-transfected cells was decreased in the presence of 1.25 $\mu$m

![Fig. 5. Effect of HBx expression on the inhibition of electron transport or uncoupling of oxidative phosphorylation in hepatoma cells. HepG2 and HepG2-3X cells were treated with 1.25 $\mu$m rotenone, 12.5 $\mu$m TTFA, 12.5 $\mu$m antimycin A, 500 $\mu$m azide, 6.25 $\mu$m oligomycin B, and 5 $\mu$m valinomycin for 24 h, and the $\Delta \Psi m$ was assessed with TMRM by flow cytometry. For the positive control, 50 $\mu$m CCCP was used.](http://www.jbc.org/Downloadedfromhttp://www.jbc.org/)
The HBx Protein Does Not Activate Apoptotic Signaling in Hepatoma Cells—Because HBx sensitizes the ΔΨm and stimulates the production of ROS, we examined whether HBx activates apoptotic signaling in hepatoma cells. As shown in Fig. 6A, HBx did not induce DNA fragmentation in HepG2-3X cells. 500 nM staurosporine-treated HepG2-3X and HepG2 cells used as a positive control showed DNA fragmentation upon treatment. Other HBx-transfected HepG2 cells (HepG2-1X and 4X) showed the same results (data not shown). Next, we examined whether HBx induces Bax expression and translocation into mitochondria. As shown in Fig. 6B-I, Bax expression was not up-regulated in HBx-transfected cells (HepG2-1X, 3X, and 4X) compared with control HepG2 cells. The translocation of Bax in these cell lines was also examined. Bax was not translocated in HBx-transfected cells and found largely in cytosolic fractions, and no detectable translocation to mitochondrial enriched heavy membranes was observed (Fig. 6B-II). These results confirmed that HBx expression does not up-regulate Bax nor translocate it into mitochondria. These results also showed that Bax was not associated with the increased production of ROS in HBx-expressing hepatoma cell lines. We then examined whether HBx is capable of regulating the release of cytochrome c from mitochondria. Immunofluorescence staining of HepG2 and HepG2-3X cells with an anti-cytochrome c antibody gave a wispy, punctate and subcytoplasmic pattern of cytochrome c.
immunostaining with no chromosomal changes, clearly showing that cytochrome c is localized in mitochondria in these cell lines (Fig. 6C, I and II). For the positive control, we treated HepG2-3X cells with 30 μM VK3 to induce apoptosis. 30 μM VK3 treatment on HepG2-3X cells induced cytoplasmic shrinkage, nuclear fragmentation, chromatin condensation observed with Hoechst staining, and a diffused cytoplasmic pattern of cytochrome c immunostaining (Fig. 6C-III), indicating that cytochrome c was translocated from mitochondria into the cytoplasm in these cell lines. To determine further whether ROS is involved in the observed cytochrome c release by apoptotic stimulus VK3, we treated cells with 2 mM glutathione (Fig. 6C-IV). As shown in Fig. 6C-IV, pretreatment with 2 mM glutathione prevented VK3-induced cytoplasmic shrinkage, nuclear fragmentation, chromatin condensation, and showed punctate cytochrome c immunostaining. The observed results demonstrated that the translocation of cytochrome c from mitochondria is dependent on ROS generation, and prevention of ROS generation blocks not only cytochrome c release from mitochondria but the induction of apoptosis. These results clearly showed that the HBx protein itself does not activate apoptotic signaling in hepatoma cells, suggesting that the amount of HBx-generated ROS is not sufficient for the release of cytochrome c, eventually causing apoptosis. Finally we examined whether HBx activates caspases by examining PARP cleavage in HepG2 and HepG2-3X, 3X, and 4X cell lines (Fig. 6D). In all cell lines PARP cleavage does not occur as determined by Western blotting using monoclonal antibody against PARP. 500 nM staurosporine-treated HepG2 cells were used as a positive control.

HBx Sensitizes Hepatoma Cells to Apoptotic Stimuli That Were Blocked by Antioxidants—The effect of HBx in HBx-expressing hepatoma cells to the response to apoptotic stimuli was examined by DNA laddering analysis, the TUNEL assay, and the ΔΨm measurement (Fig. 7, A, B, and C, respectively). Apoptosis of HepG2 and HBx-expressing HepG2 cells (1X, 3X, and 4X) was induced by treatment with VK3 (30 and 60 μM) or coucumin (30 and 60 μM) (Fig. 7A, I and II). Treatment of the...
cells with 30 μM VK3 or coucumin showed no fragmentation of DNA into oligonucleosomes in HepG2 cells, whereas HepG2-1X, 3X, and 4X cells showed fragmentation of DNA into oligomers (Fig. 7A, I and II), indicating that HBx-expressing cell lines are more sensitive to apoptotic stimuli than HepG2 cells. The antioxidant glutathione at 2 mM protected the cells from VK3-induced apoptosis in HepG2 and HepG2-3X cells (Fig. 7A-III, lanes 4 and 5), indicating that ROS are involved in VK3-induced apoptosis in these cell lines. HBx-expressing hepatoma cells (HepG2-3X) also showed strong positive staining with 30 μM VK3 treatment, whereas HepG2 cells only showed positive staining with 60 μM VK3 treatment (Fig. 7B, II and III), confirming the sensitive properties of HBx-expressing cell lines to apoptotic agents. 2 mM glutathione also blocked apoptosis in these cell lines (Fig. 7B-IV). To examine the effect of HBx and apoptotic stimuli on the ΔΨm in HBx-expressing and nonexpressing HepG2 cells, the TMRM fluorescence was measured in the presence or absence of CCCP (Fig. 7C). The VK3- and/or antioxidant-treated hepatoma cells were harvested by trypsinizing and were stained with 150 nM TMRM as described under “Experimental Procedures.” Treatment of both HepG2 and HBx-transfected HepG2 cells with CCCP demonstrated that TMRM uptake is dependent on the ΔΨm in these cell lines (HepG2 and HepG2-3X). But in HepG2-3X cells, both 30 and 60 μM VK3-treated cells decreased TMRM dye uptake, whereas 30 μM VK3 restored it (HepG2; Fig. 7C, I and II). We then added 2 mM glutathione to 60 μM VK3-treated HepG2 and 30 μM VK3-treated HepG2-3X cells and measured the TMRM dye uptake. As shown in Fig. 7C-III, both cell lines showed increased TMRM dye uptake, indicating the restoration of the ΔΨm in the normal, control cells (HepG2 and HepG2-3X). When 5 mM pyruvate was added to 60 μM VK3-treated HepG2 cells (Fig. 7C-IV), the same results as for glutathione in Fig. 6C-III were obtained. These results further confirmed the restoration of the ΔΨm in HepG2 cells in the presence of antioxidants and indicated that ROS regulate the ΔΨm in these hepatoma cell lines. Because both ROS generation and changes in the ΔΨm were observed in VK3-treated HepG2-3X cells, we measured the time course of ROS generation and the ΔΨm. The experimental results showed that HBx induced ROS generation and then ΔΨm changes which were inhibited by antioxidants, pyruvate and GSH (Fig. 8). Our results showed that ΔΨm sensitization and the decrease in HBx-transfected hepatoma cells are regulated by ROS.

**DISCUSSION**

Many different viral products are known to affect and modulate components of apoptotic signal pathways in host cells. Epstein B virus-LMP1 protein, herpes simplex virus 1, US3 protein, and human T-cell lymphotropic virus-1 tax protein interact directly with Bcl-2 family proteins in mitochondria (38, 39). The human immunodeficiency virus type 1 protein Vpr has been shown to interact directly with the mitochondrial permeability transition pore complex, producing cytochrome c release and apoptosis (40). The human HBx protein has been shown to modulate apoptosis, working as either proapoptotic (7, 41) or antiapoptotic signals (28, 42). The experimental results in our study on HBx modulations of mitochondrial enzymes, ROS production, and ΔΨm sensitization together with those of Rahmani et al. (15) could also suggest a key functional role of HBx in mitochondrial physiology and relevant functions. Several possible mechanisms could explain the HBx-induced change in mitochondrial function. HBx could alter signal transduction pathways that promote mitochondrial permeability transition, which might involve the activation of caspases, Bid and translocation of activated Bid from cytosol to mitochondria. These changes might involve mitochondrial permeability transition, release of cytochrome c, and ultimately cellular apoptosis. In contrast to the above explanations, in our system, HBx did not activate apoptotic signaling including Bax expression, Bax translocations, cytochrome c release, and caspase activation. Alternative mechanisms involving deregulated mitochondrial enzymes, sensitization of the electron transport system, and sensitization to apoptotic stimuli occur in HBx-transfected cells, which eventually induce the sensitization of mitochondrial permeability transition and production of ROS and lipid peroxide. Although sensitization of ΔΨm and ROS production occurs in HBx-induced cell lines, the exact mechanism of the correlation of ΔΨm sensitization and ROS production needs to be clarified in our system. It has been reported by Okuda et al. (18) that abnormal ROS accumulation was not blocked by treatment with cyclosporin A, an inhibitor of MPT, suggesting...
that increased MPT is not the explanation for the observed increase in ROS during apoptosis in a cellular system. However, the observation that genistein, a natural isoflavone, induces MPT by the generation of ROS by its interaction with the respiratory chain at the level of mitochondrial complex II (43) implies that MPT induction and ROS generation are directly related. In our experiment, although HBx does not directly activate the ΔΨm, the decrease of the ΔΨm by apoptotic stimuli (30 and 60 μM VK3), which was blocked by antioxidants pyruvate and GSH, shows that ROS is involved in MPT in mitochondria in our system (Fig. 7). Our results also show that mitochondria are the major source of ROS because DPI, an inhibitor of electron transport in flavoenzymes, completely abolished HBx-induced ROS production. DPI blocks mitochondrial complex I and has been shown to prevent almost all mitochondrial ROS production (44). However, DPI can also inhibit ROS production from microsomal NADH oxidase. Nonetheless, because mitochondria are the major source of cellular ROS and the HBx protein directly interacts with mitochondria, our data suggest that mitochondria are the source of HBx-induced ROS. ROS production in the mitochondrial electron transport chain is increased when the electron flow in the respiratory chain is impaired. It has been reported that blockage of electron transfer by complex inhibitors, e.g. by antimycin or cyanide or a temporary lack of the final acceptor, oxygen can lead to the increased reduction of ubiquinone and increased levels of partially reduced ubisemiquinone bound to complex I or to cytochrome bc1 (45). Some of these accumulated quinone species appear to react directly with oxygen and to be a major source of oxygen radicals (45). Because HBx induced the reduction of complex I, III, IV, and V enzyme transcripts and activities as shown in Fig. 1 and Table I, the reduced electron transfer could occur, which might be a cause of the increased reduction of ubiquinone and increased level of ubisemiquinone species bound to complex I. These accumulated quinone species could react directly with oxygen and could be a major source of oxygen radicals induced by HBx. We need to determine the status of ubiquinone species in HBx-transfected hepatoma cells as well as molecular mechanisms of unresponsiveness to HBx on complex II enzyme, succinate:ubiquinone oxidoreductase, as shown in Fig. 1 and Table I. Taken together, our results showed that HBx interacts directly with mitochondria, deregulating mitochondrial enzymes, which resulted in impairment of electron transport and thereby increasing ROS production in mitochondria. Our observations suggest a new model for the induction of apoptosis in HBx-transformed hepatoma cells as illustrated schematically in Fig. 9. Deregulated mitochondrial enzyme expression and increased abundance of ROS and lipid peroxides occur as a result of HBx protein expression, whereas ΔΨm does not change in HBx-transfected cells. This is likely to impair mitochondrial electron transport further, amplifying the effect of HBx on the mitochondria and sensitizing cells to apoptotic stimuli, implying the positive feedback effect of ROS on mitochondrial generation as proposed by Cai and Jones (46). The mechanism of ROS generation in mitochondria is quite controversial, although a recent report as mentioned above showed that ubiquinone complexes of the respiratory chain react directly with O2 and function as a major source of oxygen free radicals. Another recent report by Ricci et al. (47) showed that the rapid loss of the ΔΨm and generation of ROS are caused by activated caspases on mitochondrial electron transport complex I. But in our system, because HBx does not activate caspases, it is unlikely that caspases are involved in the loss of ΔΨm and generation of ROS. We do not know yet whether HBx interacts directly with transition metals of the respiratory chain resulting in ROS production as proposed by Salvi et al. (43) or some other mechanisms such as inducing an acceleration of Ca2+ cycling followed by the oxidation of pyridine nucleotides, eventually generating ROS (48). Because mitochondrial ROS production resulted in a catastrophic effect on the insulating properties of the inner membrane which would affect its bioenergetic capacity, the exact mechanisms of HBx involvement in ROS production resulting in lipid peroxidation need to be clarified, although we demonstrated the formation of intrahepatic lipid peroxidation in HBx transgenic mice. Cells can use a number of antioxidant mechanisms to respond to these circumstances, including the induction of antioxidant proteins that contribute to cell survival as shown in the case of HCV-core induced ROS production (18). But, in HBx-transformed cells, the expression of antioxidant genes needs to be clarified. Taken together HBx caused mitochondrial dysfunction resulting in pathophysiological conditions in HBx-expressing hepatoma cells, suggesting that HBx might be a prime candidate to mediate the pathological effect of HBV in patients with chronic hepatitis, cirrhosis, and liver cancer.

**Fig. 9. Mitochondrial injury, oxidative stress, sensitization of the ΔΨm, and deregulated gene expression in mitochondria are induced by the HBx protein.**
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Human Hepatitis B Virus-X Protein Alters Mitochondrial Function and Physiology in Human Liver Cells
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doi: 10.1074/jbc.M309280200 originally published online January 14, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M309280200

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